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531 Rec'd PCT/PT 22 OCT 2001**NEURONAL EXOCYTOSIS INHIBITING PEPTIDES AND COSMETIC AND
PHARMACEUTICAL COMPOSITIONS CONTAINING SAID PEPTIDES****FIELD OF THE INVENTION**

5 This invention refers to peptides derived from the amino end of protein SNAP-25, useful as inhibitors of neuronal exocytosis, and to their use in cosmetic and/or therapeutic applications, together (optionally) with a peptide derived from the carboxyl end of protein SNAP-25

10 BACKGROUND OF THE INVENTION

 The basis or mechanism for the formation of facial wrinkles is the tensing of the muscles of the epidermis that drag the skin inwards. This muscular tension is the result of hyperactivity of the nerves innervating the facial muscles. Nerve hyperactivity is characterized by the uncontrolled and excessive release of neurotransmitters that excite
15 muscle fibers. Because of this, the molecules that control neuronal exocytosis contribute to relaxing muscular tension, and consequently, to eliminating wrinkles.

 Botulinum toxins are a family of bacterial neurotoxins produced by *Clostridium Botulinum* (1) [see section regarding BIBLIOGRAPHY]. 7 different serotypes are known (serotypes A, B, C, D, E, F and G) with an average molecular weight of 150
20 kDa. These toxins inhibit acetylcholine exocytosis in the neuromuscular junction (nerve-muscle synapse) of the skeletal muscle (1).

 At a molecular level, botulinum toxins are proteases that degrade neuronal proteins involved in the exocytosis mechanism activated by the calcium ion (1-3). For example, botulinum toxin A, the one most commonly used clinically and cosmetically
25 [because of its applications in eliminating facial wrinkles and asymmetry, and to mitigate the symptomatology of spastic diseases], cleaves the neuronal protein SNAP-25. This protein (SNAP-25) plays a key role in neurosecretion, as it is involved in the formation of a protein complex (known as SNARE complex or fusion complex), which directs and controls the release of acetylcholine accumulated in vesicles. The nucleus of
30 said fusion complex is made up of proteins SNAP-25 and syntaxin, located in the presynaptic plasma membrane, and protein synaptobrevin (or VAMP), located in the vesicular plasma membrane (4, 5). The main function of the fusion complex is to bring the vesicle loaded with neurotransmitter (acetylcholine) nearer to the presynaptic plasma membrane and put it in contact with same (4, 5). In this way, in response to an

elevated concentration of calcium, the fusion of both plasma membranes is encouraged, thus producing the release of the neurotransmitter. Therefore, said vesicle docking and fusion protein complex (SNARE) is a key target in controlling neurosecretion. Cleaving any of the proteins that make up the fusion complex prevents its assembly, and therefore inhibits vesicle release and neuronal exocytosis.

The power of botulinum toxins and, in particular, serotype A (BOTOX®) to inhibit neurosecretion, as well as their neuronal selectivity (they only act on neurons) is being widely used therapeutically to correct spastic ailments such as dystonias, strabismus, tics, blepharospasm, facial scoliosis, etc. (6 – 13). Botulinum toxin A (botulinum A) is, moreover, an effective agent for eliminating facial wrinkles and asymmetry. In fact, the administration of BOTOX® is the first effective non-surgical therapy to eliminate the signs of aging (6, 7).

Therapeutic and cosmetic treatment with BOTOX® consists of a localized injection of diluted pharmaceutical preparations (botulinum A-hemagglutinin complex, 500 kDa) in the areas where muscular tension is localized. The paralytic effects of the toxin are reversible with an average duration of 6 months (6, 7). The treatment, therefore, requires repeated injections of BOTOX®. The main problem with this treatment is the chance that it may trigger an immune reaction against the pharmaceutical preparation due to the fact that, because of its molecular size, it may be recognized by the patient's immune system. The appearance of antibodies against botulinum A is a serious problem, as it contributes to a clear decrease in the treatment's effectiveness (6-13). This loss of effectiveness in treatment with BOTOX® means the need to increase the preparation's concentration level in later treatments, which in turn produces a potentiation of the immune response. As an alternative, the use of different botulinum toxin (BoTox) serotypes has been discussed, such as BoTox B, BoTox F and BoTox E. Nevertheless, the application of pharmaceutical preparations with different serotypes cannot be considered a solution to the problem, as sooner or later the immune reaction may once again occur. In addition, treatment with botulinum toxins is expensive, mainly because of the lability and instability of the pharmaceutical preparations containing them.

There is, therefore, a pressing need to develop molecules that imitate the paralytic effects of the botulinum toxins, but with much simpler and more stable molecular structures, which do not cause immune reactions, and whose manufacturing cost is economical. Peptide-type molecules comply with these properties.

Amino acid sequences that inhibit neuronal exocytosis have been described. Specifically, it has been proven that peptides with more than 20 amino acids, deriving from the C-terminal sequence of SNAP-25, block the release of catecholamines from permeabilized chromaffin cells (14). Likewise, peptides deriving from the amino acid sequences of proteins syntaxin and VAMP have been described that can also affect the exocytotic process (15). Although these peptides show biological activity, their later development as cosmetic and/or therapeutic agents has not occurred, most likely due to their size, as this complicates their development as useful therapeutic agents and makes it more expensive. Therefore, there is a need to find molecules of a smaller size that can be applied in cosmetics and medicine.

This invention provides a solution to the existing needs which includes the discovery of smaller amino acid sequences, between 3 and 30 amino acids, deriving from the amino end (N-terminal domain) of protein SNAP-25, which inhibit neuronal exocytosis. In addition, it has been discovered that the simultaneous use of peptides deriving from the amino end and from the carboxyl end (C-terminal domain) of SNAP-25 produces a considerable increase in their inhibitory activity, i.e., there is a potentiation of their activity compared to that shown by individual peptides.

Therefore, one object of this invention is a peptide that has a sequence made up of 3 to 30 adjacent amino acids contained on the amino end of protein SNAP-25, which inhibits neuronal exocytosis.

An additional object of this invention is a nucleic acid that essentially codes for one of the peptides provided by this invention. The plasmids and vectors that contain said nucleic acid (also identified as constructions), as well as the cells transformed with said plasmids or vectors that express a peptide of the invention, also constitute additional objects of this invention.

Another additional object of this invention is a mixture of at least one of the peptides provided by this invention and at least one peptide that has a sequence made up of 3 to 30 adjacent amino acids contained on the carboxyl end of protein SNAP-25.

Another additional object of this invention is a cosmetic composition that includes at least one of the peptides provided by this invention. The use of the peptides provided by this invention in the preparation of said cosmetic composition, as well as the method of cosmetic treatment that includes the application of said cosmetic composition, constitute additional objects of this invention.

Another additional object of this invention is a pharmaceutical composition that includes at least one of the peptides provided by this invention, or alternatively, a vector containing a nucleic acid that codes for one of the peptides of the invention. The use of the peptides and vectors (constructions) provided by this invention in the preparation of said pharmaceutical compositions, as well as the method of treating humans or animals encompassed by the application of said cosmetic composition, constitute additional objects of this invention.

Another additional object of this invention is a combination of drugs that includes at least one of the peptides provided by this invention, along with, at least, one drug intended for a second therapeutic target which may be the same as or different from the therapeutic target at which the peptide provided by this invention is aimed.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a peptide deriving from the amino end of protein SNAP-25. More specifically, the invention provides a peptide, henceforth known as the peptide of the invention, which has a sequence of 3 to 30 adjacent amino acids contained in SEQ. ID. No. 1 [see the section regarding the SEQUENCE LIST].

The invention also includes peptides which are substantially homologous to the peptide of the invention. In the sense used in this description, the expression "substantially homologous" means that the peptide in question has a homology level, as far as amino acids are concerned, of at least 60%, and preferably of at least 80%, and even more preferably, of at least 95%.

The invention also includes peptides which are functionally equivalent to the peptide of the invention. In the sense used in this description, the expression "functionally equivalent" means that the peptide in question has at least one of the biological activities of the peptide of the invention, such as, for example, the ability to at least partially inhibit neuronal exocytosis.

In one particular embodiment, the peptide of the invention has a length of 3 to 20 amino acids, and preferably from 6 to 19 amino acids.

The amino acids that make up the structural units of the peptide of the invention may have D- or L-configuration. The amino acid from the amino end may have an acetylated terminal amino group, and the amino acid from the carboxyl end may have an amidated terminal carboxyl group. Therefore, this invention also includes

derivatives of the peptide of the invention in which the amino-terminal end is acetylated and/or in those where the carboxy-terminal end is amidated.

Particular examples of peptides of the invention are those peptides that have sequences of amino acids shown in SEQ. ID No. 2 and SEQ. ID No. 3.

5 Within the scope of this invention are cosmetically and/or pharmaceutically acceptable salts of the peptide of the invention. The term "cosmetically and/or pharmaceutically acceptable salts" includes salts customarily used to form metal salts or salts formed by adding free acids or bases. The nature of the salt is not critical, as long as it is cosmetically and/or pharmaceutically acceptable. Cosmetically and/or
10 pharmaceutically acceptable salts of the peptide of the invention may be obtained from acids or bases, organic or inorganic, by conventional methods which are well known to technicians in these matters, by making the appropriate acid or base react with the peptide of the invention.

In addition, the peptide of the invention may undergo reversible chemical
15 modifications in order to increase its bioavailability (including stability and fat solubility) and its ease in passing through the blood-brain barrier and epithelial tissue. Examples of such reversible chemical modifications include the esterification of the carboxylate groups of glutamic and aspartic amino acids with an acetyl-methyl group, by which the negative charge of the amino acid is eliminated and its hydrophobicity is
20 increased. This esterification is reversible, as the ester link formed is recognized by intracellular esterases which hydrolyze it, giving back the charge to the aspartic and glutamic residues. The net effect is an accumulation of intracellular peptide, as the internalized, de-esterified peptide cannot cross the cell membrane.

The peptide of the invention can be obtained through conventional methods for
25 solid-phase chemical peptide synthesis, following Fmoc and/or Boc-based methodology (16).

Alternatively, the peptide of the invention can be obtained through conventional methods based on recombinant DNA technology, e.g., through a method that, in brief, includes inserting the nucleic acid sequence that codes for the peptide of the invention
30 into an appropriate plasmid or vector, transforming competent cells for said plasmid or vector, and growing said cells under conditions that allow the expression of the peptide of the invention and, if desired, isolating and (optionally) purifying the peptide of the invention through conventional means known to experts in these matters. The nucleic acid sequence that codes for the peptide of the invention may be easily deduced from

the correspondence that exists between the amino acids and the nucleotide codons that code for such amino acids. In this case, an additional object of the invention is an isolated nucleic acid sequence that codes for the peptide of the invention. In one particular embodiment, said nucleic acid is selected among single-strand DNA, double-stranded DNA, and RNA. Additional objects of this invention are the plasmids and expression vectors that contain said nucleic acid sequence that codes for the peptide of the invention, as well as prokaryotic or eukaryotic cells that express the peptide of the invention. A review of the principles of recombinant DNA technology may be found, for example, in the text book entitled "*Principles of Gene Manipulation: An Introduction to Genetic Engineering*," R.W. Old & S.B. Primrose, published by Blackwell Scientific Publications, 4th Edition (1989).

The peptide of the invention is able to at least partially inhibit neuronal exocytosis, probably through a mechanism that involves interfering with the assembly of the fusion protein complex (SNARE) and/or its thermal destabilization.

The neuronal-exocytosis (neurosecretion) inhibiting capabilities of the peptides of the invention became evident through a test that evaluates the strength of said peptides in inhibiting the release of catecholamines induced by calcium in chromaffin cells permeabilized with a detergent [see Example 1.2.1]. Briefly, the chromaffin cell cultures are incubated with epinephrine and norepinephrine containing tritium, are permeabilized with digitonin, and stimulated with calcium, and the amount of radioactivity released by the cells to the extracellular medium, which is a reflection of the exocytosis of said tritium-containing catecholamines, is measured.

The hexapeptide of the invention [SEQ. ID. No. 2], at a concentration of 1 mM, blocked approximately 20% of the release of catecholamines (epinephrine and norepinephrine) in permeabilized chromaffin cells, while the peptide with 13 amino acids [SEQ. ID. No. 3], at a concentration of 1 mM, inhibited approximately 35% of the release of catecholamines in the permeabilized chromaffin cells.

The peptides shown in SEQ. ID. No. 5 and SEQ. ID. No. 6, from the carboxyl end of SNAP-25 [SEQ. ID. No. 4], inhibited the secretion induced by Ca^{2+} in chromaffin cells permeabilized with digitonin by approximately 40% when they were used at a concentration of 1mM.

Parallel tests performed using, jointly, at least one peptide from the amino end of SNAP-25, for example, the peptide of SEQ. ID. No. 2 or of SEQ. ID. No. 3, and at least one peptide from the carboxyl end of SNAP-25, for example, the peptide of SEQ. ID.

No. 5 or of SEQ. ID. No. 6, made it evident that the combined use of at least one peptide from the amino end of SNAP-25 and at least one peptide from the carboxyl end of SNAP-25 strengthens the biological activity observed for each of the peptides tested separately.

5 In one particular case, mixtures of peptides made up of one of the peptides shown in SEQ. ID. No. 2 or in SEQ. ID. No. 3 and one of the peptides shown in SEQ. ID. No. 5 or in SEQ. ID. No. 6, at a concentration of 0.5 mM for each of them, were tested, and an inhibition rate of 55% was obtained in the release of catecholamines in permeabilized chromaffin cells.

10 Taken all together, these results indicate that both types of peptides, both those from the amino end and those from the carboxyl end, inhibit catecholamine exocytosis, and that the combined use of peptides from the amino end and the carboxyl end strengthens the biological activity observed for each of them separately.

Therefore, the invention also provides a mix of peptides which includes:

- 15 (a) at least one peptide of the invention, and
(b) at least one peptide with a sequence of 3 to 30 adjacent amino acids contained in SEQ. ID. No. 4 [henceforth, (COOH) peptide to indicate its relationship with the carboxyl end of SNAP-25].

In one particular embodiment, said mix of peptides is made up of at least one peptide selected from the group formed by the peptides shown in SEQ. ID. No. 2 and in SEQ. ID. No. 3, and at least one peptide selected from the group formed by the peptides shown in SEQ. ID. No. 5 and in SEQ. ID. No. 6.

The ability of the peptides of the invention to interfere with the formation and stability of the fusion complex (SNARE) became evident through the performance of *in vitro* reconstitution tests of the fusion protein complex with recombinant proteins [see Example 1.2.2]. Briefly, protein SNAP-25 was immobilized in 96-well plates, proteins VAMP and syntaxin were added in the presence and/or absence of the peptides of the invention, and the formation of the fusion protein complex (SNARE) was evaluated. The detection of the complex was performed using an antibody against syntaxin (anti-syntaxin), followed by a second, tagged antibody which recognizes the anti-syntaxin antibody. The data obtained seem to indicate that the presence of the peptides of the invention during the assembly of the fusion complex causes a significant decrease in same. Therefore, the mechanism of the neuronal exocytosis inhibiting action seems to

imply that the peptides of the invention interfere with the formation and/or stability of the fusion protein complex (SNARE).

The results obtained with said tests suggest that the peptides of the invention, peptides which are small in size, between 3 and 30 amino acids, deriving from the amino acid sequence from the amino end of SNAP-25, along with (optionally) peptides from the carboxyl end of SNAP-25, act as neuronal exocytosis inhibitors. Given that these peptides imitate the sequences of neuronal proteins involved in exocytosis, their activity is specific, as they only interact with the corresponding neuronal proteins without affecting other cell components.

The action mechanism of the peptides of the invention seems to be similar to that of botulinum toxins, thus affecting the formation and/or stability of the fusion protein complex; so the peptides of the invention can be considered to have cosmetic/therapeutic applications similar to those described for botulinum toxin. Therefore, the peptides of the invention can become effective, stable, safe and economical substitutes for botulinum toxins, both in the treatment of facial wrinkles and/or asymmetry and in the treatment of the symptomatology of spastic diseases, and their use as neuroprotectors in the treatment of neurological disorders and neurodegenerative diseases.

The studies made by the applicants suggest, moreover, the innovative concept of the simultaneous use of peptides from the N-terminal and C-terminal domains of SNAP-25 as neuronal exocytosis modulators.

Taken together, the results obtained with the peptides of the invention, along with their stability and structural simplicity and the chemical diversity that can be obtained, keeping in mind the composition of the amino and carboxyl ends of SNAP-25, give the peptides of the invention great cosmetic and/or therapeutic potential.

The peptides of the invention may be used for pathological neuronal exocytosis-mediated cosmetic and/or therapeutic purposes.

Among the cosmetic applications of the peptides of the invention are the treatment and total or partial elimination of facial wrinkles and/or asymmetry in humans.

The invention provides a cosmetic composition that includes a cosmetically effective amount of at least one peptide of the invention, along with at least one cosmetically acceptable adjuvant. Additionally and optionally, said cosmetic composition may contain one of the peptides identified as (COOH) peptide.

For cosmetic applications, the peptides of the invention may be applied through any medium that produces contact between the peptide and the place where it is to act in a mammal's body, preferably in humans.

The cosmetically effective amount of peptide that should be applied, as well as the dosage for the treatment of facial wrinkles and/or asymmetry with the peptides and/or cosmetic compositions of the invention, will depend on numerous factors, including the age and condition of the person desiring treatment, the severity of the wrinkles and/or facial asymmetry, the method and frequency of application and the particular peptide to be used.

The presentation of the cosmetic compositions containing the peptides of the invention may be in any form that is suitable for application, e.g., solid, liquid or semi-solid, such as creams, ointments, gels or solutions, and the application of these compositions may be by any suitable means, preferably topically, so they will include the cosmetically acceptable adjuvants necessary to make up the desired form of administration. In a preferred and particular embodiment, the peptides of the invention are encapsulated in liposomes, along with (optionally) another or other (COOH) peptide(s), which are added to the other components of the cosmetic preparation. A review of the different cosmetic forms for applying active compounds and of the adjuvants necessary for obtaining same may be found, for example, in the text book "Cosmetología de Harry" (Harry's Cosmetology), Wilkinson & Moore, Ed. Díaz de Santos (1990).

Therefore, an additional object of this invention is the use of the peptides of the invention in the preparation of cosmetic compositions for the treatment of facial wrinkles and/or asymmetry.

The invention also provides a method for the cosmetic treatment of facial wrinkles and/or asymmetry in mammals, preferably humans, which consists of applying a cosmetically effective amount of at least one peptide of the invention to the mammal that has facial wrinkles and/or asymmetry, along with (optionally) one or more (COOH) peptides, preferably in the form of a cosmetic composition containing it.

In addition, the peptides of the invention are suitable for the treatment of spastic diseases, for example, dystonias, strabismus, blepharospasm, facial scoliosis, tics, etc.; and/or as neuroprotectors in the treatment of neurological disorders and/or neurodegenerative diseases.

Among said neurological disorders are acute neurological diseases, for example, those that take place in the first stages of cerebral ischemia. It is a known fact that during an ischemic process an uncontrolled release of the neurotransmitter glutamate takes place in the affected area. This neurotransmitter interacts with specific neuronal
5 membrane receptors causing a massive influx of calcium ions inside the neuron. The intracellular calcium causes the release of more glutamate, thus triggering a chain reaction. Moreover, the massive, prolonged influx of calcium inside the neurons causes their death, which translates into the formation of necrotic tissue in the ischemic zone. Clearly, the progress of the ischemic damage can be stopped, at least partially, if the
10 uncontrolled glutamate exocytosis is controlled. Therefore, the peptides of the invention, because of their ability to inhibit exocytosis, may be suitable for preventing and/or slowing down the neuronal death that is characteristic of an ischemic process, and so would be useful in the treatment of neuropathologies that occur because of excessive glutamate exocytosis, such as, for example, senile dementia, Alzheimer's-
15 related dementia, AIDS-related dementia, epilepsy, amiotrophic sclerosis, multiple/lateral sclerosis, etc. In this case, application in the treatment of neurological diseases would be similar to the one described for botulinum toxin A (18).

The peptides of the invention could therefore form part of a combined therapy (aimed at several therapeutic targets) with the objective of more effectively stopping
20 neurodegeneration.

An additional object of this invention is a pharmaceutical composition which includes a therapeutically effective amount of at least one peptide of the invention, along with at least one pharmaceutically acceptable excipient. In one particular embodiment, said pharmaceutical composition also contains one or more (COOH)
25 peptides. Alternatively, the pharmaceutical composition of the invention may contain a therapeutically effective amount of a vector that contains at least one nucleic acid sequence that codes for a peptide of the invention, along with at least one adjuvant and/or a pharmaceutically acceptable excipient. Said vector may be used in gene therapy.

30 The active products of the invention (peptides or vectors) may be administered for the treatment of pathological neuronal exocytosis, manifested, for example, by spastic diseases, neurological disorders or neurodegenerative diseases, through any medium that produces contact between the peptide and the place where it is to act in a mammal's body, preferably in humans.

The therapeutically effective amount of the active product of the invention [peptides or vectors (constructions)] that should be administered, as well as the dosage for the treatment of a pathological condition with the peptides and/or pharmaceutical compositions of the invention, will depend on numerous factors, including the age and condition of the patient, the severity of the disturbance or disorder, the method and frequency of administration and the particular peptide to be used.

The presentation of the pharmaceutical compositions that contain the peptides or vectors (constructions) of the invention may be in any form that is suitable for administration, e.g., solid, liquid or semi-solid, such as creams, ointments, gels or solutions, and these compositions may be administered by any suitable means, for example, orally, parenterally or topically, so they will include the pharmaceutically acceptable excipients necessary to make up the desired form of administration. A review of the different pharmaceutical forms for administering medicines and of the excipients necessary for obtaining same may be found, for example, in the "Tratado de Farmacia Galénica" (Treatise on Galenic Pharmacy), C. Faulí i Trillo, 1993, Luzán 5, S.A. Ediciones, Madrid.

As was previously mentioned, the peptides of the invention could form part of a combined therapy for the purpose of more effectively stopping neurodegeneration. In this case, the invention provides a pharmaceutical composition that includes at least one peptide of the invention, along with (optionally) another or other neuronal-exocytosis inhibiting compound(s), and along with at least one drug intended for another therapeutic target, selected from the group formed by a neuronal glutamate receptor blocker, a calcium chelator, an anti-oxidant, a free-radical destroyer and their combinations.

In one particular embodiment, said composition that is useful in combined therapy may contain at least one peptide of the invention, along with (optionally) another or other neuronal exocytosis inhibiting compound(s) and a neuronal glutamate receptor blocker. In another embodiment of this invention, said composition could contain at least one peptide of the invention, along with (optionally) another or other neuronal exocytosis inhibiting compound(s), a neuronal glutamate receptor blocker, a calcium chelator, an anti-oxidant and/or a free-radical destroyer. Among the neuronal exocytosis inhibiting compounds are peptides from the carboxyl end of SNAP-25, identified as (COOH) peptides. Many other examples of compositions may be proposed, all having in common the need to control neurotransmitter exocytosis.

An additional object of this invention is the use of the peptides of the invention or of vectors that contain at least one sequence that codes for a peptide of the invention, in the preparation of a medicine for the treatment of pathological neuronal exocytosis-mediated pathological diseases and/or disorders, such as, for example, spastic diseases, neurological disorders and/or neurodegenerative diseases.

In addition, the invention provides a method for the treatment in mammals of pathological neuronal exocytosis-mediated pathological diseases and disorders such as, for example, spastic diseases, neurological disorders and/or neurodegenerative diseases, which consists of administering to said mammal suffering from said pathological disease or disorder a therapeutically effective amount of at least one peptide of the invention, or of a vector containing at least one DNA sequence that codes for a peptide of the invention, preferably in the form of a pharmaceutical composition that contains it. In one particular embodiment of this invention, said pharmaceutical composition contains, in addition to the peptide or peptides of the invention, one or more (COOH) peptides.

The following examples serve to illustrate the nature of this invention and should not be considered in a restricting sense as regards said invention.

EXAMPLE 1

Neurotransmitter exocytosis inhibiting peptides

1.1 Peptide synthesis

The peptides shown in SEQ. ID. No. 2, SEQ. ID. No. 3, SEQ. ID. No. 5 and SEQ. ID. No. 6 have been synthesized through conventional methods for solid-phase chemical peptide synthesis using Fmoc and/or Boc-based synthetic methodology (16).

The resulting peptides were purified by high-performance liquid chromatography (HPLC) and were analyzed by mass spectrometry.

1.2 Evaluation of biological activity

To evaluate the biological activity of the peptides obtained in Example 1.1, a test was developed that evaluates the strength of said peptides in inhibiting the release of catecholamines induced by calcium in chromaffin cells, as well as an *in vitro* reconstitution test of the fusion complex (SNARE).

1.2.1 Inhibition of the release of catecholamines

This test was performed to verify the neuronal exocytosis-inhibiting capabilities of the peptides synthesized in Example 1.1. In this test, the strength of said peptides is evaluated in inhibiting the release of catecholamines (norepinephrine and epinephrine) induced by calcium in chromaffin cells (obtained from suprarenal bovine glands) permeabilized with the detergent digitonin, in accordance with the method described by Gutiérrez *et al.* (1995 and 1997).

Briefly, the chromaffin cell cultures are incubated with [³H] -epinephrine and [³H] -norepinephrine, are permeabilized with 20 μM digitonin, and stimulated with calcium (10 μM), in the presence of the peptides to be tested (separate or mixed), and the amount of radioactivity released by the cells to the extracellular medium, which is a reflection of the exocytosis of [³H] -epinephrine and [³H] -norepinephrine, is measured.

The results obtained in inhibiting the release of catecholamines in permeabilized chromaffin cells were the following:

a) the peptide in SEQ. ID. No. 2, from the amino end of SNAP-25, at a concentration of 1 mM, blocked approximately 20% of the release of catecholamines in permeabilized chromaffin cells;

b) the peptide in SEQ. ID. No. 3, from the amino end of SNAP-25, at a concentration of 1 mM, inhibited approximately 35% of the release of catecholamines in the permeabilized chromaffin cells.

c) the peptides in SEQ. ID. No. 5 and SEQ. ID. No. 6, from the carboxyl end of SNAP-25, at a concentration of 1mM, inhibited the secretion induced by Ca²⁺ in chromaffin cells permeabilized with digitonin by approximately 40%; and

d) mixtures of peptides made up of one of the peptides shown in SEQ. ID. No. 2 or in SEQ. ID. No. 3 and one of the peptides shown in SEQ. ID. No. 5 or in SEQ. ID. No. 6, at a concentration of 0.5 mM for each of them, inhibited the release of catecholamines in permeabilized chromaffin cells by approximately 55%.

Taken together, these results indicate that both types of peptides, both those from the amino end and those from the carboxyl end, inhibit catecholamine exocytosis, and that the combined use of peptides from the amino end and the carboxyl end strengthens the biological activity observed for each of them separately.

1.2.2 In vitro reconstitution

This test was performed to determine the ability of the peptides obtained in Example 1.1 to interfere with the formation and stability of the fusion complex (SNARE). The test consists of evaluating the *in vitro* reconstitution of the fusion protein complex with recombinant proteins produced in *Escherichia coli*. The reconstitution tests, based on ELISA (Enzyme-Linked Immuno Assay) methods, involve the immobilization of protein SNAP-25 in 96-well plates and the subsequent formation of the fusion protein complex by adding the proteins VAMP and syntaxin in the presence and/or absence of the peptides of the invention. The detection of the complex was performed using an antibody against protein syntaxin (anti-syntaxin), followed by an antibody which recognizes the anti-syntaxin antibody, covalently tagged with a peroxidase. The amount of fusion protein complex was tracked by adding 1,2-phenylenediamine dichloride, whose reaction with the peroxidase produces a product with an orangish-yellow color that absorbs 492 nm in an acid medium.

The data obtained show that the presence of the peptides obtained in Example 1.1 during the assembly of the fusion complex causes a significant decrease in same. Therefore, the mechanism of the action of said peptides seems to imply that said peptides interfere with the formation and/or stability of the fusion protein complex (SNARE).

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